

## Cloned Calves from Chromatin Remodeled In Vitro<sup>1</sup>

Eddie J. Sullivan,<sup>4</sup> Sriranjani Kasinathan,<sup>4</sup> Poothappillai Kasinathan,<sup>4</sup> James M. Robl,<sup>3,4</sup> and Philippe Collas<sup>2,5</sup>

Hematech,<sup>4</sup> LLC, Sioux Falls, South Dakota 57106

Institute of Medical Biochemistry,<sup>5</sup> University of Oslo, Oslo 0317, Norway

### ABSTRACT

We have developed a novel system for remodeling mammalian somatic nuclei in vitro prior to cloning by nuclear transplantation. The system involves permeabilization of the donor cell and chromatin condensation in a mitotic cell extract to promote removal of nuclear factors solubilized during chromosome condensation. The condensed chromosomes are transferred into enucleated oocytes prior to activation. Unlike nuclei of nuclear transplant embryos, nuclei of chromatin transplant embryos exhibit a pattern of markers closely resembling that of normal embryos. Healthy calves were produced by chromatin transfer. Compared with nuclear transfer, chromatin transfer shows a trend toward greater survival of cloned calves up to at least 1 mo after birth. This is the first successful demonstration of a method for directly manipulating the somatic donor chromatin prior to transplantation. This procedure should be useful for investigating mechanisms of nuclear reprogramming and for making improvements in the efficiency of mammalian cloning.

early development, embryo

### INTRODUCTION

Limitations to the application of mammalian cloning technology by nuclear transplantation (NT) are low rates of embryonic development, high rates of pregnancy loss, and low survival of cloned offspring [1–3]. Substantial effort has been put toward characterizing defects in cloned embryos and offspring; however, information gathered from these studies [2–4] has not yet yielded any novel approaches for improving survival of clones.

Extracts from gametes or somatic cells have been used to investigate the dynamics of the cell nucleus at fertilization or during the cell cycle [5, 6]. Notably, extracts from mitotic cells have been shown to support disassembly of exogenously added nuclei, including nuclear envelope breakdown and chromosome condensation [7–9]. In vitro nuclear disassembly is compatible with subsequent reconstitution of functional nuclei [9]. Moreover, somatic cell extracts containing nuclear and cytoplasmic components have been developed to alter chromatin organization and gene expression in exogenous nuclei [10] or in permeabilized somatic cells [11].

The objective of this study was to determine whether cloned animals could be produced after manipulating the somatic donor nucleus in vitro prior to transfer into a recipient oocyte. We report that disassembly of somatic nuclei in a mitotic extract followed by transfer of the resulting condensed chromatin into oocytes enhances nuclear remodeling. Chromatin transfer (CT) is compatible with development to term and shows trends of improved viability of cloned animals.

### MATERIALS AND METHODS

#### *In Vitro-Produced Embryos*

In vitro fertilization was performed, and embryos were cultured as described [12, 13].

#### *Nuclear Transplantation and Oocyte Activation*

NT was carried out by fusing donor bovine fetal fibroblasts to enucleated oocytes [1, 13]. Metaphase stage NTs (M-NTs) were performed in nocodazole-containing media (1  $\mu$ g/ml) using donor fibroblasts synchronized in M phase with 1  $\mu$ g/ml nocodazole for 18 h. Recipient oocytes were activated at 28–30 h postmaturation (hpm) with 5  $\mu$ M calcium ionophore for 4 min followed by 10  $\mu$ g/ml cycloheximide (CHX) and 2.5  $\mu$ g/ml cytochalasin D for 5 h and washed, and embryos were cocultured with mouse fetal fibroblasts [13]. For CHX treatment, oocytes were activated as above and embryos were cultured with 2.5  $\mu$ g/ml CHX for another 9 h before culture. For actinomycin D (ActD) treatment, oocytes were activated as above except that 5  $\mu$ g/ml ActD was added to the 5-h CHX incubation step and embryos were maintained in 5  $\mu$ g/ml ActD for another 9 h prior to culture.

#### *Mitotic Extract*

Madin-Darby bovine kidney (MDBK) cells (American Type Culture Collection, Bethesda, MD) were synchronized in mitosis with 0.75–1  $\mu$ g/ml nocodazole for 18 h, harvested by mitotic shake off, and washed twice in phosphate buffered saline and once in cell lysis buffer (20 mM Hepes, pH 8.2, 5 mM MgCl<sub>2</sub>, 10 mM EDTA, 1 mM dithiothreitol, and a cocktail of protease inhibitors) [7]. Sedimented cells were resuspended in 1 volume of ice-cold cell lysis buffer, swollen on ice for 1 h, and Dounce-homogenized using a tight-fitting glass pestle. The lysate was centrifuged at 15 000  $\times$  g for 15 min at 4°C, and the supernatant (mitotic extract) was aliquoted, frozen in liquid nitrogen, and stored at –80°C. Fresh or frozen extracts were used without noticeable differences on the efficiency of nuclear breakdown.

#### *Chromatin Transfer*

In vitro-matured oocytes were enucleated at 20 hpm. Bovine fetal fibroblasts from confluent cultures were washed in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free Hank's Balanced Salt Solution (HBSS; Gibco-BRL; Invitrogen, Carlsbad, CA) and permeabilized by incubation of 100 000 cells in suspension with 31.2 U Streptolysin O (SLO; Sigma, St. Louis, MO) in 100  $\mu$ l HBSS for 30 min in a 37°C H<sub>2</sub>O bath. Permeabilization was assessed by uptake of the membrane impermeant DNA stain, propidium iodide (0.1  $\mu$ g/ml). Permeabilized fibroblasts were sedimented, washed, and incubated in 40  $\mu$ l mitotic extract containing an ATP-generating system (1 mM ATP, 10 mM creatine phosphate, and 25  $\mu$ g/ml creatine kinase) for 45 min at 38°C. Aliquots were labeled with 0.1  $\mu$ g/ml Hoechst 33342 to monitor chromatin condensation. At the end of incubation, the reaction mix was diluted with

<sup>1</sup>Supported by Aurox, LLC, the Norwegian Research Council, and the Research Council of Norway.

<sup>2</sup>Correspondence: Philippe Collas, Institute of Medical Biochemistry, University of Oslo, P.O. Box 1112 Blindern, 0317 Oslo, Norway. FAX: 47 2285 1058; e-mail: philippe.collas@basalmed.uio.no

<sup>3</sup>Correspondence: James M. Robl, Aurox, LLC, 33 Riverside Avenue, 2nd Floor, Westport, CT 06880. e-mail: jrobl@hematech.com

Received: 14 July 2003.

First decision: 20 August 2003.

Accepted: 2 September 2003.

© 2004 by the Society for the Study of Reproduction, Inc.

ISSN: 0006-3363. http://www.biolreprod.org

**This Page Blank (uspto)**

---

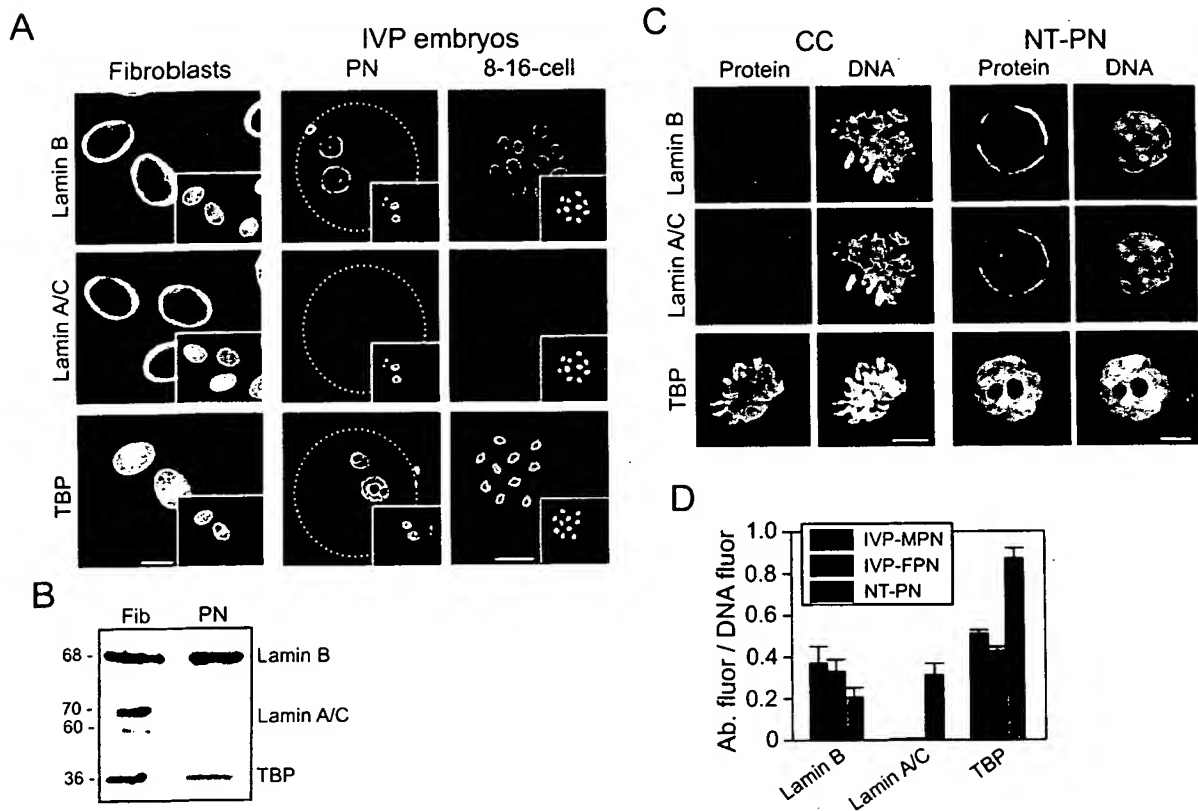


FIG. 1. Dynamics of fibroblast nuclei after NT. A) Immunofluorescence distribution of lamin B, lamin A/C, and TBP in bovine fibroblasts and IVP pronuclear (PN), and 8–16-cell stage bovine embryos. Insets = DNA labeled with Hoechst 33342; bars = (left) 10  $\mu$ m, (right) 50  $\mu$ m. B) Immunoblot of lamin B, lamin A/C, and TBP in fetal fibroblasts and pronuclear embryos. C) Immunofluorescence analysis of lamin B, lamin A/C, and TBP at condensed chromosome (CC) and pronuclear (NT-PN) stages in NT embryos. Bars = 10  $\mu$ m. D) Immunolabeling intensity of indicated proteins in MPN and FPN of IVP embryos and in pronuclei of NT embryos (NT-PN). Data are expressed as mean  $\pm$  SD ratio of antibody fluorescence over Hoechst 33342 (DNA) fluorescence intensity. More than 20 embryos/markers were analyzed in 3–5 replicates in (A, C, D).

500  $\mu$ l Alpha MEM/10% fetal bovine serum (Gibco-BRL) containing 2 mM  $\text{CaCl}_2$  for membrane resealing, and cells were cultured for 2 h at 38.5°C. Resealing was monitored by propidium iodide (0.1  $\mu$ g/ml) uptake. Resealed cells were fused to enucleated oocytes, oocytes were activated at 28 hpm, and embryos were cultured as described for NT.

### Embryo Transfer

NT and CT embryos were cultured to the blastocyst stage in vitro, and two embryos were transferred per synchronized recipient female. Pregnancies were monitored by ultrasonography, and C-sections were performed by independent veterinarians. All animal work was performed following a protocol approved by the Trans Ova Genetics (Sioux Center, IA) institutional animal care and use committee.

### Immunological Procedures

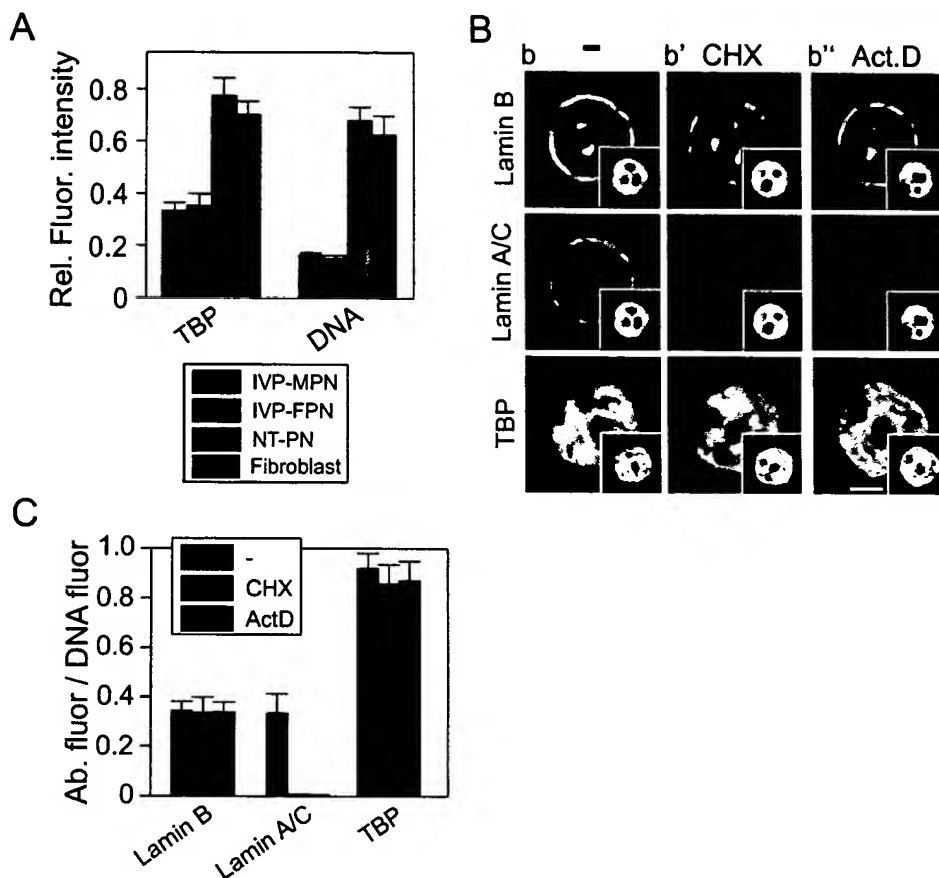
Goat polyclonal antibodies against B-type lamins (referred to as "lamin B" in this paper), anti-lamin A/C monoclonal antibodies, and anti-TBP polyclonal or monoclonal antibodies were from Santa-Cruz Biotechnology (Santa Cruz, CA). Anti-AKAP95 antibodies were from Upstate Biotechnologies (Lake Placid, NY). Immunofluorescence analysis was performed as described [14]. Briefly, cells and embryos were settled onto poly-L-lysine-coated coverslips, fixed with 3% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 15 min, and proteins were blocked in PBS/2% BSA/0.01% Tween 20. Samples each were incubated with primary and secondary antibodies (1:100 dilutions) for 30 min. DNA was counterstained with 0.1  $\mu$ g/ml Hoechst 33342. Photographs were taken with a JVC CCD camera, and quantification of immunofluorescence intensity was performed using the Analysis software. When indicated, samples were extracted on coverslips with a cocktail of 1% Triton X-100, 1 mg/ml DNase I, and 300 mM NaCl in Tris-HCl (pH 7.2) for 15 min prior to fixation and immunofluorescence analysis. For immunoblotting, protein samples (30  $\mu$ g) were resolved by 10% SDS-PAGE, blotted onto nitrocellulose, and probed with indicated antibodies [14].

## RESULTS

### Behavior of the Donor Nucleus in Nuclear Transplant Embryos

We first investigated the dynamics of components of somatic nuclei during bovine NT. We examined the distribution of nuclear lamins, intermediate filament proteins of the nuclear envelope, and intranuclear skeleton. B-type lamins are ubiquitously expressed [15]. A-type lamins (which include the splice variants lamins A and C) are restricted to differentiated cells [15] and would not be expected to be expressed in preimplantation NT embryos. Lamins anchor nuclear membranes to chromatin and may promote nuclear expansion after nuclear reconstitution in vitro [16, 17]. Furthermore, mutations in the lamin A (*LMNA*) gene cause life-threatening diseases in humans [15], suggesting that lamins may be involved in the regulation of gene expression. Nuclear lamins are also essential for cell survival, as failure to assemble B-type lamins leads to cell death [18]. As a nonhistone component of chromatin and as a marker of the transcription machinery, the dynamics of the TATA-binding protein, TBP, a transcription factor for virtually all genes [19], was also analyzed. Perinuclear distribution of lamin B and colocalization of TBP with DNA in bovine fetal fibroblasts and in in vitro-produced (IVP) preimplantation embryos were consistent with observations in other species [20–22] (Fig. 1A). Lamin A/C was not detected during preimplantation development (Fig. 1A) as expected from a marker of differentiated cells. Immunofluorescence data were corroborated on Western blots (Fig. 1B).

FIG. 2. Characterization of pronuclei in NT embryos. **A**) Immunolabeling intensity (mean  $\pm$  SD) of TBP and DNA (Hoechst 33342) in pronuclear IVP and NT embryos and in fibroblasts after in situ extraction with 1% Triton X-100/1 mg/ml DNase I/300 mM NaCl. Labeling intensity is expressed relative to that of nonextracted embryos ( $n = 30$  embryos or cells per group). **B**) NT embryos were activated as (b) described in the text, or (b') in the presence of 10  $\mu$ g/ml CHX or (b'') 5  $\mu$ g/ml ActD ( $n = 30$  embryos/group in 2–3 replicates). Insets = DNA; bar = 10  $\mu$ m. **C**) Ratio (mean  $\pm$  SD) of immunolabeling intensity of indicated proteins over Hoechst 33342 (DNA) fluorescence in embryos activated as in **B** ( $n = 15$ –20 embryos/treatment/marker).



Within 3 h of transplantation of fibroblast nuclei into enucleated oocytes, donor chromosomes condensed and excluded both lamins A/C and B immunolabeling, whereas TBP remained associated with the chromosomes (Fig. 1C, CC). Fourteen hours after initiation of recipient oocytes activation, all NT embryos contained fully developed pronuclei with perinuclear lamin B labeling and TBP colocalized with DNA (Fig. 1C, NT-PN). However, in contrast to IVP embryos, 95–99% of NT embryos also displayed lamin A/C expression at the pronuclear stage (Fig. 1C), and expression persisted during early development (see below). Relative amounts of immunolabeled lamin B, lamin A/C, and TBP in pronuclei of NT and IVP embryos were quantified by measuring the ratio of secondary antibody fluorescence intensity to that of DNA (Hoechst 33342) to account for DNA content (haploid versus diploid) in the nuclei examined. Whereas relative amounts of lamin B were similar in pronuclei of NT and IVP embryos, relative amounts of lamin A/C and TBP were higher in NT pronuclei than in male (MPN) or female (FPN) pronuclei of IVP embryos (Fig. 1D).

#### *TBP, DNA, and A-type Lamins in Pronuclei of NT Embryos*

Higher amounts of TBP in NT pronuclei were associated with a greater resistance to in situ extraction with a combination of detergent (1% Triton X-100), nuclease (1 mg/ml DNase I), and salt (0.3 M NaCl). Quantification of immunofluorescence labeling intensity in extracted embryos relative to that of nonextracted controls shows that ~35% of TBP remained unextracted in MPN or FPN of IVP embryos (Fig. 2A). However, TBP of NT pronuclei displayed strong resistance to extraction as in fibroblast nuclei (Fig.

2A). Similarly, DNA of NT pronuclei displayed a 4.5-fold increase in resistance to extraction under these conditions compared with pronuclei of IVP embryos (Fig. 2A, DNA). Altogether, the results suggest a more compact chromatin organization in pronuclei of NT embryos compared with IVP embryos.

To determine the origin of lamin B, lamin A/C, and TBP in pronuclei of NT embryos, recipient oocytes were activated in the presence of the RNA polymerase (Pol) II inhibitor ActD (5  $\mu$ g/ml) or with the protein synthesis inhibitor CHX (10  $\mu$ g/ml) [23]. Assembly of lamin A/C, lamin B, and TBP was examined by densitometric analysis of immunofluorescently labeled pronuclear embryos. Both inhibitors prevented pronuclear lamin A/C assembly (Fig. 2, B and C), suggesting that assembly of these somatic lamins in NT embryos resulted from transcription of the lamin A gene at the pronuclear stage. Lamin B assembly was not perturbed by CHX or ActD treatment (Fig. 2, B and C), suggesting that it was assembled from somatic lamins solubilized in the oocyte cytoplasm after NT and/or from a maternal pool of lamins. Similar amounts of TBP were detected in untreated embryos or after inhibition of RNA or protein synthesis (Fig. 2, B and C). Note that in IVP embryos, TBP remains undetectable (our unpublished data) until the pronuclear stage when it is upregulated (see Fig. 1A). Thus presumably TBP associated with condensed chromosomes in NT embryos represents a carry-over from the somatic nucleus, and TBP detected in NT pronuclei represents carry-over from the somatic nucleus in addition to any pronuclear stage embryonic production.

#### *A Chromatin Transfer Strategy*

In an attempt to alleviate defects identified in pronuclear NT embryos, we developed a procedure for directly ma-

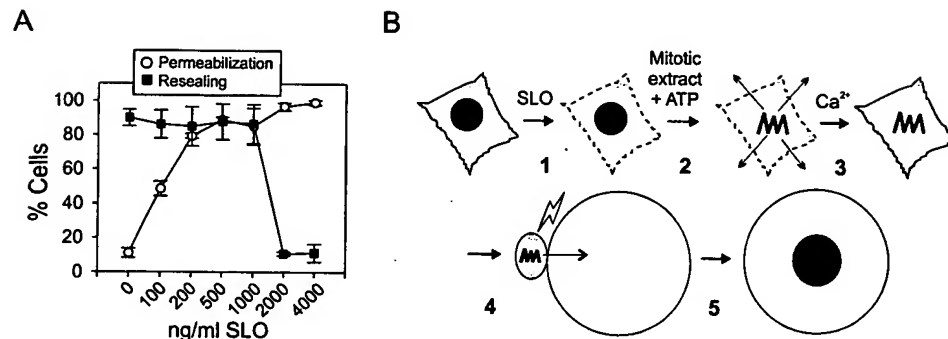


FIG. 3. Cloning by CT. A) Efficiency of SLO-mediated permeabilization and subsequent  $\text{Ca}^{2+}$ -mediated resealing of cultured bovine fetal fibroblasts, as determined by the proportion of cells taking up the membrane-impermeant DNA stain, propidium iodide. B) CT procedure: 1) Donor fibroblasts are reversibly permeabilized for 30 min with 500 ng/ml SLO; 2) permeabilized cells are washed and incubated for 45 min at 38°C in a mitotic extract containing an ATP-regenerating system to elicit chromosome condensation and promote removal of nuclear components (arrows); 3) extract is removed and cells resealed in culture with 2 mM  $\text{CaCl}_2$  for 2 h; 4) resealed cells are fused to enucleated recipient oocytes; and 5) oocytes are activated as for NT to elicit pronuclear formation and development.

nipulating donor fibroblast nuclei *in vitro* prior to transfer into recipient oocytes. Fibroblasts were permeabilized with 500 ng/ml of the bacterial toxin Streptolysin O (SLO), as judged by uptake of the membrane-impermeant DNA stain, propidium iodide (Fig. 3A). Permeabilized fibroblasts were incubated in a mitotic extract for 45 min at 38°C to promote nuclear disassembly and removal of nuclear components (Fig. 3B; see below). The fibroblasts were recovered from the extract by sedimentation, washed, and cultured for 2 h with 2 mM  $\text{CaCl}_2$  to reseal the plasma membrane [11] (Fig. 3, A and B). The resealed fibroblasts containing condensed chromosomes (as opposed to intact interphase nuclei; see below) were fused to recipient oocytes (Fig. 3B), and oocytes were activated with calcium ionophore/CHX/cytochalasin D as described for NT.

#### Breakdown of Fibroblast Nuclei in Mitotic Extract

The mitotic extract consisted of a 15 000 g supernatant from a lysate of mitotic MDBK cells and contained an ATP-regenerating system. The extract did not induce apoptosis, as judged by the absence of proteolysis of poly(ADP)ribosyl polymerase (PARP) and DNA fragmentation characteristic of apoptotic fibroblasts (Fig. 4A), and thus was suitable to promote remodeling of nuclei.

The extract elicited ATP-dependent condensation of chromosomes, disassembly of A- and B-type lamins from chromatin (as judged by immunolabeling of these lamins distributed throughout the cytoplasm), and removal of TBP from chromatin (Fig. 4B). These events were confirmed by immunoblotting analysis of condensed chromatin purified from the fibroblasts after recovery from the mitotic extract (Fig. 4C; compare lanes 1 and 3). In this experiment, the A-kinase anchoring protein AKAP95 [7] was used as a marker of a nuclear component that remains associated with the condensed chromosomes, as normally occurs at mitosis and upon chromosome condensation *in vitro* [7]. Histone H4 was used as a protein loading control in the gel (Fig. 4C). Disassembly of nuclear lamins and TBP from chromatin in mitotic extract was dependent on an ATP-regenerating system (Figs. 4B and 4C, lanes 3 and 4) and was reminiscent of that occurring in mitotic cells (Fig. 4C, lane 2). Furthermore, immunoblotting analysis of whole permeabilized fibroblasts (as opposed to isolated chromatin fractions) after exposure to the mitotic extract showed that a proportion of solubilized lamin A/C and all detectable TBP were eliminated from the cells and/or proteolyzed

(Fig. 4C, lane 6). Finally, a control extract from interphase fibroblasts (Fig. 4C, lane 5) or cell lysis buffer alone (not shown), both containing an ATP-regenerating system, failed to promote nuclear disassembly, indicating that nuclear breakdown was specific for the mitotic extract. Permeabilized fibroblasts exposed to mitotic extract and subsequently resealed with 2 mM  $\text{CaCl}_2$  in the culture medium could be cultured over several passages (data not shown). Thus membrane permeabilization, incubation of the permeabilized cells in the mitotic extract, and membrane resealing produced viable cells.

#### Characterization of Nuclei in Embryos Produced by Chromatin Transfer

Extract-treated and resealed fibroblasts were fused to recipient oocytes as efficiently (>70%) as nonpermeabilized control cells. The donor chromatin was in a condensed form at the time of introduction into the oocyte (Fig. 5A, CT). In contrast, chromatin of untreated control fibroblasts used for NT was still decondensed within 30 min of fusion (Fig. 5A, NT). Thus resealing of mitotic extract-treated fibroblasts with  $\text{CaCl}_2$  prior to transfer into oocytes did not promote nuclear reformation in the donor cells. This observation was supported by the absence of a nuclear envelope around the condensed chromatin in CT embryos immediately after fusion, as judged by immunofluorescence analysis of lamina and inner nuclear membrane proteins (data not shown).

Immunolabeling of nuclear lamins and TBP in nuclei of CT and NT embryos, and immunolabeling intensity of these markers relative to DNA fluorescence intensity, are shown in Figure 5, B and C. Perinuclear lamin B labeling intensity was similar in CT and NT pronuclei. However, in contrast to NT embryos, lamin A/C was undetected in pronuclei and up to at least the 8–16-cell stage in CT embryos. CT pronuclei also displayed a fourfold reduction in TBP labeling compared with NT pronuclei (Fig. 5C). Pronuclear TBP concentration in the mouse has been shown to increase during progression through interphase [22]. However, as kinetics of pronuclear formation from donor chromatin condensed in the oocytes or from *in vitro*-condensed chromatin were similar in NT and CT embryos, respectively (unpublished data), it is unlikely, albeit not formally excluded, that enhanced TBP concentration in NT pronuclei was due to a more advanced cell cycle stage. Resistance of TBP to extraction with 1% Triton X-100, 1 mg/ml DNase I, and 0.3

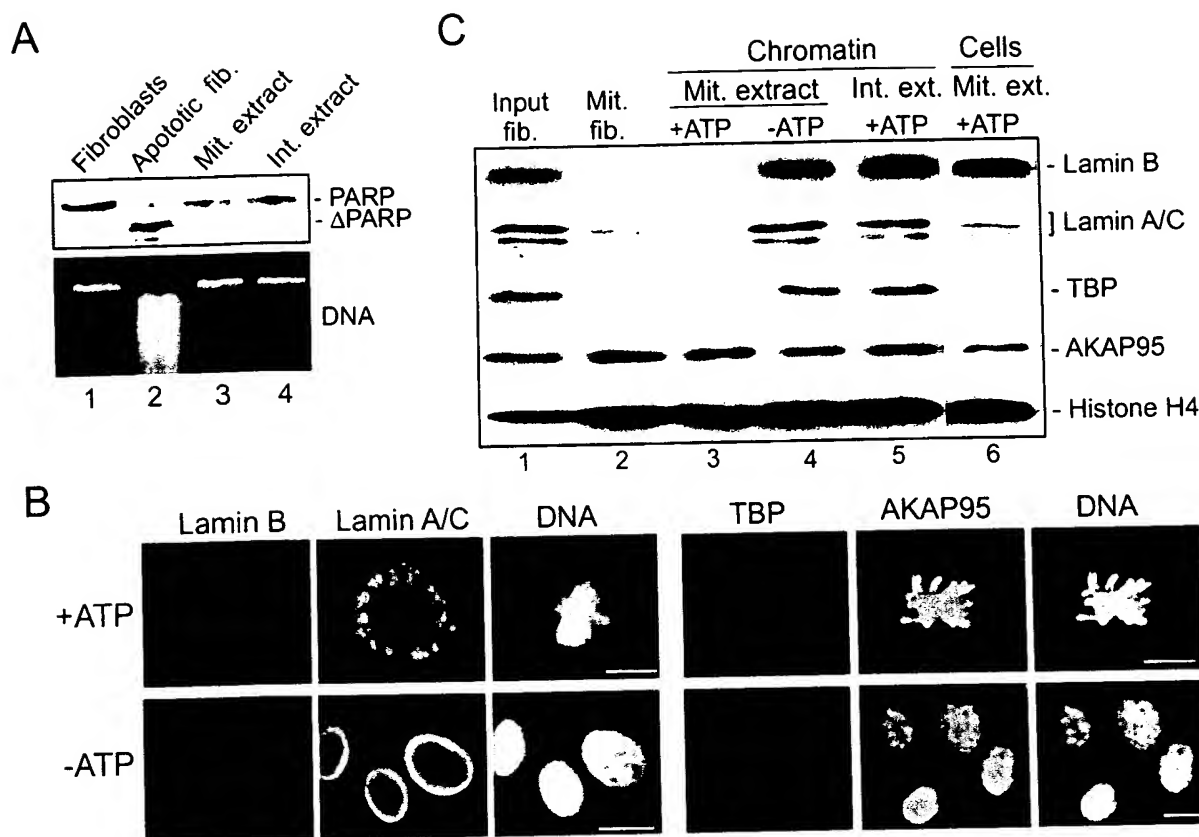


FIG. 4. Somatic nuclear breakdown in mitotic extract. A) Mitotic extract does not elicit apoptosis. Intact bovine fibroblasts (lane 1), fibroblasts induced into apoptosis with 5  $\mu$ g/ml nocodazole for 20 h (lane 2), or SLO-permeabilized fibroblasts exposed to mitotic (lane 3) or interphase (lane 4) extract for 1 h were immunoblotted using anti-PARP antibodies (upper panel).  $\Delta$ PARP indicates proteolyzed PARP. DNA degradation was evaluated by gel electrophoresis in 0.8% agarose and staining with ethidium bromide (lower panel). B) Immunofluorescence analysis of lamin B, lamin A/C, TBP, and AKAP95 after a 45-min incubation of permeabilized fibroblasts in mitotic extract with (+ATP) or without (-ATP) an ATP-regenerating system. Bars = 10  $\mu$ m. Over 1000 cells analyzed in four replicates displayed consistent labeling as shown. C) Immunoblotting analysis interphase fibroblasts (lane 1); fibroblasts synchronized at mitosis with 1  $\mu$ g/ml nocodazole (lane 2); chromatin isolated from permeabilized fibroblasts exposed to mitotic extract with or without the ATP-regenerating system (lanes 3, 4) or to interphase extract (lane 5); and whole permeabilized fibroblasts exposed to mitotic extract (lane 6). Blots were probed using antibodies to indicated proteins.

M NaCl was decreased by more than twofold (Fig. 5D), suggesting a weaker association of TBP with chromatin. Likewise, resistance of DNA to DNase I was reduced nearly fourfold in CT pronuclei, suggesting that CT favors the establishment of a looser chromatin configuration in pronuclei (Fig. 5D).

Transfer into oocytes of fibroblasts synchronized in M-phase with nocodazole resulted in pronuclei with lamin A/C and CT clones (11.2% [63/506 recipients] vs. 15.4% [42/273 recipients], respectively,  $P > 0.1$ ). Furthermore, whereas the proportion of live calves at birth did not differ for NT and CT cloned animals (9% [46/506 recipients] vs. 10% [27/273 recipients], respectively,  $P > 0.1$ ) across the six cell lines tested, the proportion of animals alive and healthy at 1 mo postpartum tended to be higher for CT (8.4%, 23/273 recipients) than for NT (5.1%, 26/506 recipients;  $P = 0.07$ ; Fig. 6, A and B). Notably, however, CT did not obviously eliminate variations in development between cell lines cloned. Mean birth weight of CT and NT clones was not significantly different (data not shown). Altogether, our results indicate that CT produces live offspring and shows trends of improved viability of clones.

#### Chromatin Transfer Produces Clones

CT resulted in development to term of cloned embryos (Fig. 6). In vitro development to blastocysts was similar for

CT embryos (661/5880 cultured embryos, 11.2%) and NT embryos (1154/9362, 12.3%;  $P > 0.1$ ). These figures represent data from six different bovine fetal fibroblast cell lines cloned both by CT and NT within an 18-month period. Notably, both CT and NT displayed a similar variation in the efficiency of development to blastocysts between cell lines (data not shown). Likewise, pregnancy rates (not shown) and proportions of calves born following transfer of blastocysts into recipient females were similar for NT and CT clones (11.2% [63/506 recipients] vs. 15.4% [42/273 recipients], respectively,  $P > 0.1$ ). Furthermore, whereas the proportion of live calves at birth did not differ for NT and CT cloned animals (9% [46/506 recipients] vs. 10% [27/273 recipients], respectively,  $P > 0.1$ ) across the six cell lines tested, the proportion of animals alive and healthy at 1 mo postpartum tended to be higher for CT (8.4%, 23/273 recipients) than for NT (5.1%, 26/506 recipients;  $P = 0.07$ ; Fig. 6, A and B). Notably, however, CT did not obviously eliminate variations in development between cell lines cloned. Mean birth weight of CT and NT clones was not significantly different (data not shown). Altogether, our results indicate that CT produces live offspring and shows trends of improved viability of clones.

#### DISCUSSION

This is the first report of manipulation of a somatic nucleus in a cell extract as a step to enhance subsequent nu-

**BEST AVAILABLE COPY**

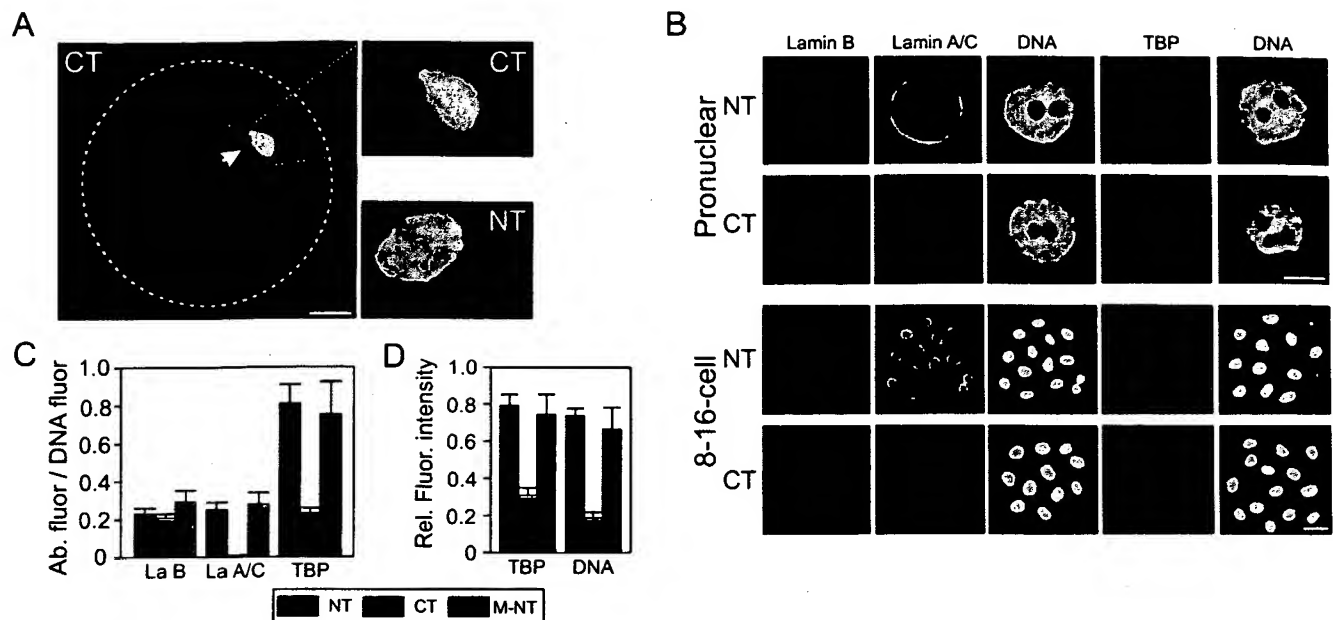


FIG. 5. Characterization of nuclei of CT embryos. A) Chromatin morphology (arrow) within 30 min of introduction into an oocyte by CT or NT, as indicated. Dotted line delineates the oocyte cytoplasm. Panels on the right show enlargements of donor chromatin in a CT and NT embryo. Bar = 20  $\mu$ m. B) Distribution of lamin B, lamin A/C, and TBP in pronuclear (upper two panels) and 8- to 16-cell-stage (lower two panels) embryos produced by NT or CT. Bars = 20  $\mu$ m ( $n$  = 20–30 embryos analyzed per marker in three replicates). C) Ratio (mean  $\pm$  SD) of immunolabeling intensity over Hoechst 33342 (DNA) fluorescence intensity for indicated protein in pronuclei of NT, CT, or M-NT embryos ( $n$  ~ 20 embryos/group/marker). D) Immunolabeling intensity (mean  $\pm$  SD) of TBP and DNA (Hoechst 33342) in pronuclei of NT, CT, and M-NT embryos after extraction with 0.1% Triton X-100/1 mg/ml DNase I/300 mM NaCl relative to that in nonextracted embryos ( $n$  = 15–30 embryos/group).

clear remodeling in the oocyte and efficiency of mammalian cloning. Permeabilization of the donor cell, induction of nuclear breakdown in a mitotic extract, and membrane resealing produces viable cells. The birth of cloned calves produced by CT supports this contention. The overall efficiency of producing cloned calves by CT appears similar to NT. Nevertheless, CT exhibits a trend toward enhanced survival of cloned calves at 1 mo postpartum. Moreover, in vitro breakdown of the somatic nucleus creates opportunities for directly accessing and manipulating the donor genome prior to introduction into the recipient oocyte. Remodeling of the somatic chromatin was demonstrated by induction of condensation of chromosomes in the mitotic extract. This clearly is distinct from a normal mitotic chromosome condensation, since condensation was elicited in fibroblasts from confluent cultures and therefore not in a G2 phase. One might anticipate possibilities for altering DNA methylation in donor cells prior to cloning to, tentatively, correct methylation defects in cloned animals [24–26]. Similarly, histone modifications might also be manipulated in vitro prior to cloning. Epigenetic manipulations of the donor genome might, speculatively, lead to enhanced development and health of clones.

We have identified several nuclear defects in NT embryos, including assembly of lamin A/C, enhanced pronuclear TBP content, and increased resistance of DNA to DNase I. Our results complement recent observations in primate NT embryos resulting from somatic cell cloning, which display defects in mitotic spindle organization [27]. Abnormalities we observed may result from incomplete remodeling of the fibroblast nuclei and/or from misregulation of expression of differentiated cell-specific (e.g., lamin A) genes. Remodeling of nuclei in vitro and transplantation of condensed chromatin into oocytes alleviates these defects. Interestingly, however, transfer of M-phase cells into oocytes results in pronuclei resembling NT pronuclei with re-

spect to lamin A/C and TBP expression and TBP anchoring. Thus chromosome condensation per se is not sufficient to rescue the defects at the pronuclear stage, suggesting that the cycle stage at which chromosome condensation takes

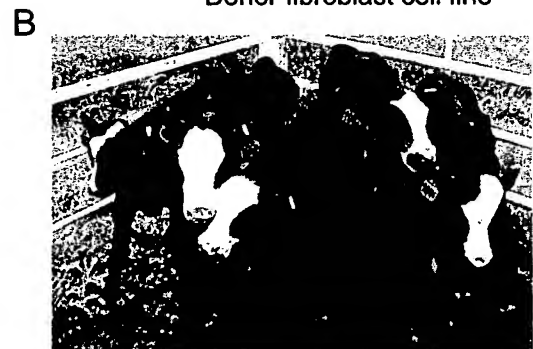
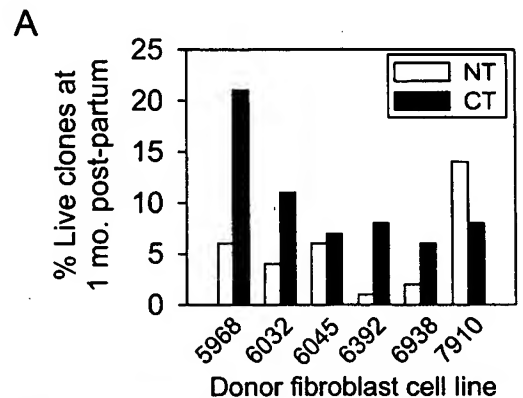


FIG. 6. Development of CT and NT clones. A) Percentages of live clones produced by NT and CT 1 mo after birth (percentage of recipient cows). See text for details. B) CT clones (Simmental  $\times$  Angus cross identical females) at 5–6 wk of age.



place may be an important factor. A hypothesis is that in vitro remodeling through condensation of chromosomes during interphase alters the "memory" of chromatin organization in the somatic nuclei, which mere passage through a timely mitosis (i.e., after G2 phase) does not achieve [28]. This hypothesis remains to be tested.

Remodeling nuclei through CT increases DNA sensitivity to DNase I and may promote the formation of transcriptionally active (or potentially active) chromatin. This may, in turn, facilitate expression of developmentally important genes. It will be interesting to identify and investigate the regulation of genes involved in placental development, maintenance of late pregnancy, and postnatal survival. CT also induces repression of lamin A gene expression in cloned embryos. In vitro and in vivo manipulations of nuclear lamina composition have shown that failure to assemble a correct set of lamins invariably leads to apoptosis [18]. Moreover, as lamins interact with DNA, chromatin, and the transcription machinery, proper lamina reconstitution is likely to be essential for normal nuclear function [15, 29] in cloned embryos.

Chromatin condensation at mitosis or in vitro is associated with the release of DNA-bound components such as chromatin remodeling enzymes [30], transcription factors (such as TBP), or other potentially inhibitory somatic components. TBP removal from somatic nuclei was also induced in interphase *Xenopus* egg extracts as a result of ATP-dependent SWI-SNF-complex activity, but was not due to chromosome condensation [31]. It is not clear whether loss of TBP from fibroblast nuclei in our study results from chromosome condensation or SWI-SNF complex-related activities. In any event, TBP release from donor nuclei is more efficient under mitotic conditions (in vitro or during mitosis; Fig. 4) than under meiotic conditions (Fig. 1C). Resulting reduced TBP concentration in CT pronuclei, however, reflects dynamic transitions in nuclear structure and function [31]. In particular, removal of TBP from donor somatic chromatin may facilitate the repression or down-regulation of somatic-specific genes in CT embryos, which may impair development. An implication of removing factors from the donor nucleus is that loading of maternal components onto chromatin and subsequent remodeling into a physiological pronucleus may be facilitated.

In conclusion, we demonstrate that it is possible to remodel a somatic nucleus in a cell extract and produce live offspring. In vitro manipulation of nuclei for cloning or transdifferentiation purposes [10, 11] may constitute a useful tool for investigating the mechanisms of nuclear reprogramming. CT shows a trend toward improved viability of clones. Additional manipulation of the system might lead to further improvements in the efficiency of mammalian cloning.

## ACKNOWLEDGMENTS

We are grateful to M. Nichols, M. Ahlers, M. Bien, J. Griffin, and T. King (Hematech) for technical assistance, and D. Faber, J. Pommer, J. Koster, J. Molina, and others (Trans Ova Genetics; Sioux Center, IA) for animal evaluation and care.

## REFERENCES

- Kasinathan P, Knott JG, Wang Z, Jerry DJ, Robl JM. Production of calves from G1 fibroblasts. *Nat Biotechnol* 2001; 19:1176–1178.
- Edwards JL, Schrick FN, McCracken MD, Van Amstel SR, Hopkins FM, Welborn MG, Davies CJ. Cloning adult farm animals: a review of the possibilities and problems associated with somatic cell nuclear transfer. *Am J Reprod Immunol* 2003; 50:113–123.
- Wilmot I, Beaujean N, De Sousa PA, Dinnyes A, King TJ, Paterson LA, Wells DN, Young LE. Somatic cell nuclear transfer. *Nature* 2002; 419:583–586.
- Cibelli JB, Campbell KH, Seidel GE, West MD, Lanza RP. The health profile of cloned animals. *Nat Biotechnol* 2002; 20:13–14.
- Collas P, Poccia DL. Methods for studying in vitro assembly of male pronuclei using oocyte extracts from marine invertebrates: sea urchins and surf clams. *Meth Cell Biol* 1998; 53:417–452.
- Poccia DL, Collas P. Nuclear envelope dynamics during male pronuclear development. *Devel Growth Differ* 1997; 39:541–550.
- Collas P, Le Guellec K, Tasken K. The A-kinase anchoring protein, AKAP95, is a multivalent protein with a key role in chromatin condensation at mitosis. *J Cell Biol* 1999; 147:1167–1180.
- Martins SB, Eide T, Steen RL, Jahnsen T, Skålhegg BS, Collas P. HA95 is a protein of the chromatin and nuclear matrix regulating nuclear envelope dynamics. *J Cell Sci* 2000; 113:3703–3713.
- Steen RL, Martins SB, Tasken K, Collas P. Recruitment of protein phosphatase 1 to the nuclear envelope by A-kinase anchoring protein AKAP149 is a prerequisite for nuclear lamina assembly. *J Cell Biol* 2000; 150:1251–1262.
- Landsverk HB, Håkelién AM, Küntziger T, Robl JM, Skålhegg BS, Collas P. Reprogrammed gene expression in a somatic cell-free extract. *EMBO Rep* 2002; 3:384–389.
- Håkelién AM, Landsverk HB, Robl JM, Skålhegg BS, Collas P. Reprogramming fibroblasts to express T-cell functions using cell extracts. *Nat Biotechnol* 2002; 20:460–466.
- Collas P, Sullivan EJ, Barnes FL. Histone H1 kinase activity in bovine oocytes following calcium stimulation. *Mol Reprod Dev* 1993; 34:224–231.
- Kasinathan P, Knott JG, Moreira PN, Burnside AS, Jerry DJ, Robl JM. Effect of fibroblast donor cell age and cell cycle on development of bovine nuclear transfer embryos in vitro. *Biol Reprod* 2001; 64:1487–1493.
- Bomar J, Moreira P, Balise JJ, Collas P. Differential regulation of maternal and paternal chromosome condensation in mitotic zygotes. *J Cell Sci* 2002; 115:2931–2940.
- Gruenbaum Y, Wilson KL, Harel A, Goldberg M, Cohen M. Review: nuclear lamins—structural proteins with fundamental functions. *J Struct Biol* 2000; 129:313–323.
- Collas P, Pinto-Correia C, Poccia DL. Lamin dynamics during sea urchin male pronuclear formation in vitro. *Exp Cell Res* 1995; 219:687–698.
- Jenkins H, Hölman T, Lyon C, Lane B, Stick R, Hutchison C. Nuclei that lack a lamina accumulate karyophilic proteins and assemble a nuclear matrix. *J Cell Sci* 1995; 106:275–285.
- Steen RL, Collas P. Mistargeting of B-type lamins at the end of mitosis: implications on cell survival and regulation of lamins A/C expression. *J Cell Biol* 2001; 153:621–626.
- Sharp PA. TATA-binding protein is a classless factor. *Cell* 1992; 68:819–821.
- Holy J, Wessel G, Berg L, Greg RG, Schatten G. Molecular characterization and expression patterns of a B-type nuclear lamin during sea urchin embryogenesis. *Dev Biol* 1995; 168:464–478.
- Houliston E, Guilly MN, Courvalin JC, Maro B. Expression of nuclear lamins during mouse preimplantation development. *Development* 1988; 102:271–278.
- Worrad DM, Ram PT, Schultz RM. Regulation of gene expression in the mouse oocyte and early preimplantation embryo: developmental changes in Sp1 and TATA box-binding protein, TBP. *Development* 1994; 120:2347–2357.
- Knott JG, Poothapillai K, Wu H, He CL, Fissore RA, Robl JM. Porcine sperm factor supports activation and development of bovine nuclear transfer embryos. *Biol Reprod* 2002; 66:1095–1103.
- Bourc'his D, Le Bourhis D, Patin D, Niveleau A, Comizzoli P, Renard J, Viegas-Pequignot E. Delayed and incomplete reprogramming of chromosome methylation patterns in bovine cloned embryos. *Curr Biol* 2001; 11:1542–1546.
- Chung YG, Ratnam S, Chaillet JR, Latham KE. Abnormal regulation of DNA methyltransferase expression in cloned mouse embryos. *Biol Reprod* 2003; 69:146–153.
- Dean W, Santos F, Reik W. Epigenetic reprogramming in early mammalian development and following somatic nuclear transfer. *Semin Cell Dev Biol* 2003; 14:93–100.



27. Simerly C, Dominko T, Navara C, Payne C, Capuano S, Gosman G, Chong KY, Takahashi D, Chace C, Compton D, Hewitson L, Schatten G. Molecular correlates of primate nuclear transfer failures. *Science* 2003; 300:297.
28. Gerlich D, Beaudouin J, Kalbfuss B, Daigle N, Eils R, Ellenberg J. Global chromosome positions are transmitted through mitosis in mammalian cells. *Cell* 2003; 112:751–764.
29. Cohen M, Lee KK, Wilson KL, Gruenbaum Y. Transcriptional repression, apoptosis, human disease and the functional evolution of the nuclear lamina. *Trends Biochem Sci* 2001; 26:41–47.
30. Sif S, Stukenberg PT, Kirschner MW, Kingston RE. Mitotic inactivation of a human SWI/SNF chromatin remodeling complex. *Genes Dev* 1998; 12:2842–2851.
31. Kikyo N, Wade PA, Guschin D, Ge H, Wolffe AP. Active remodeling of somatic nuclei in egg cytoplasm by the nucleosomal ATPase ISWI. *Science* 2000; 289:2360–2362.

This Page Blank (uspto)